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14. ABSTRACT

Our laboratory research is focused on the potential roles of a TNF-related cytokine named TWEAK and its specific cell surface receptor named Fn14 in tumor biology. We reported previously that the Fn14 gene is highly expressed in many human breast cancers. In this Breast Cancer Concept Award application we proposed to investigate whether we could make Fn14-targeted toxins that would kill Fn14-positive breast cancer cells in vitro and in vivo. Our most significant findings during the research period are that although TWEAK-based single chain or two chain non-covalently linked toxins do have some cytotoxicity on cancer cells this approach is not ideal because of protein aggregation issues, likely problems with translating these molecules to clinical use, and the recent identification of a second TWEAK-binding protein. A different approach, namely using an Fn14 mAb named ITEM4 to deliver toxic cargo, appears to be a better strategy. Indeed, we have shown that both ITEM4-gelonin chemical conjugates and ITEM4/gelonin-based single chain proteins have cytotoxic activity on Fn14-positive breast cancer cells.

15. SUBJECT TERMS
TWEAK, Fn14, breast cancer, immunotoxin

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Introduction: Breast cancer kills ~ 570,000 women per year in the USA and thus it is critical that we identify new therapeutic agents. Our laboratory research is focused on the potential roles of a TNF-related cytokine named TWEAK and its specific cell surface receptor named Fn14 in tumor biology (the TWEAK-Fn14 axis is reviewed in reference 1) We reported previously that the Fn14 gene is highly expressed in many human breast cancers (2). In this Breast Cancer Concept Award application we proposed to investigate whether we could make Fn14-targeted toxins that would kill Fn14-positive breast cancer cells *in vitro* and *in vivo*. If so, this could be a new therapeutic approach for certain breast cancer patients.

Body:
Specific Aim 1: To determine whether a TWEAK-cytotoxin fusion protein or noncovalent complex has apoptotic activity on Fn14+ but not Fn14- breast cancer cells cultured *in vitro*.

Task Summary: Construct expression plasmids, purify proteins, test proteins for cytotoxic effects on breast cancer cell lines.

Progress: The majority of the results summarized below and under Specific Aim 2 were obtained in collaboration with Dr. Michael Rosenblum's group at the University of Texas MD Anderson Cancer Center. Our initial plan was to prepare TWEAK-cytotoxin fusion proteins in which TWEAK serves as the targeting moiety and either *Pseudomonas* exotoxin PE38 protein or a recombinant form of a plant toxin named gelonin (denoted rGel) act as the cytotoxic cargo. We found that these fusion proteins were difficult to purify from *E. coli* due to aggregation problems. In addition, when unaggregated protein was tested for its ability to kill Fn14-positive breast cancer cells the cytotoxic effect was not impressive (see Annual Report). Another approach listed in this Aim was to express and purify a protein A-Fc binding domain:rGel fusion protein, mix it with an IgG-Fc:TWEAK fusion protein (provided by Dr. Schneider, Univ. of Lausanne) and then add the noncovalent complex to cells. We did see some cytotoxicity on MDA-MB-231 breast cancer cells when these proteins were added at either a 3:1 or 2:1 ratio (see Annual Report). However, this approach, which requires mixing two proteins together, would probably not be applicable to patient treatment because the non-covalent complex may dissociate during IV administration or during transit in the bloodstream. Also, during the course of these studies a manuscript was published indicating that TWEAK can bind a monocyte/macrophage protein named CD163 (3). The identification of a second TWEAK-binding cell surface protein (besides Fn14) suggested to us that using TWEAK to direct toxins to Fn14+ tumor cells may not be the best strategy. An alternative strategy is to use an Fn14 extracellular domain-specific monoclonal antibody to deliver toxins into Fn14+ cells, and these studies are described below.

We have developed two types of Fn14-targeted immunotoxins, both of which are based on the anti-Fn14 monoclonal antibody named ITEM4. This antibody was provided to us by Dr. Yagita (Juntendo University, Tokyo, JP). In our initial experiments, ITEM4 was chemically conjugated to gelonin. This ITEM4-rGel conjugate binds the Fn14 receptor and has excellent cytotoxic activity on many tumor cell lines, including breast cancer cell lines. This work has been published (4) and therefore will not be described in detail here. The manuscript is included in the Appendix. Chemical conjugates are not ideal for patient therapy because of their bulky size and other properties; therefore, we next constructed several single-chain immunotoxins. The structure of the Fn14 immunotoxin designated $_{h}$scFvIT4/rGel/29 is shown in **Fig. 1A.** The N-terminal region consists of a single-chain Fv (scFv) fragment of ITEM4 in which the V_H and V_L sequences from the hypervariable region were linked by a flexible peptide. These two domains were "humanized" by site-specific mutagenesis to alter certain amino acid residues within the framework domain without impacting affinity or specificity. The C-terminal region consists of rGel followed by a short dimerization domain to produce a bivalent immunotoxin. The $_{h}$scFvIT4/rGel/29 and rGel proteins were expressed in bacteria and purified. We then tested for cytotoxic activity using the human HER2+/Fn14+ MCF-7/HER2 and AU565 breast cancer cell

lines and a mouse embryonic fibroblast (MEF) cell line derived from Fn14-null mice. We should note here that the ITEM4 antibody recognizes both human and mouse Fn14. The $_h$scFvIT4/rGel/29 protein had cytotoxic activity on the two breast cancer cell lines but not the MEF cell line (**Fig. 1B).** The IC_{50} values were: MCF-7/HER2= 12.5 nM, AU565= 1.3 nM, MEF= 352 nM. The targeting index, defined as the IC_{50} of rGel divided by the IC_{50} of $_h$scFvIT4/rGel/29, was 21.4 for MCF-7/HER2, 570 for AU565, and <1 for MEF 3.5-/-. The precise mechanisms responsible for tumor cell killing by $_h$scFvIT4/rGel/29 are currently under investigation and appear to be specific to the particular cell type under study.

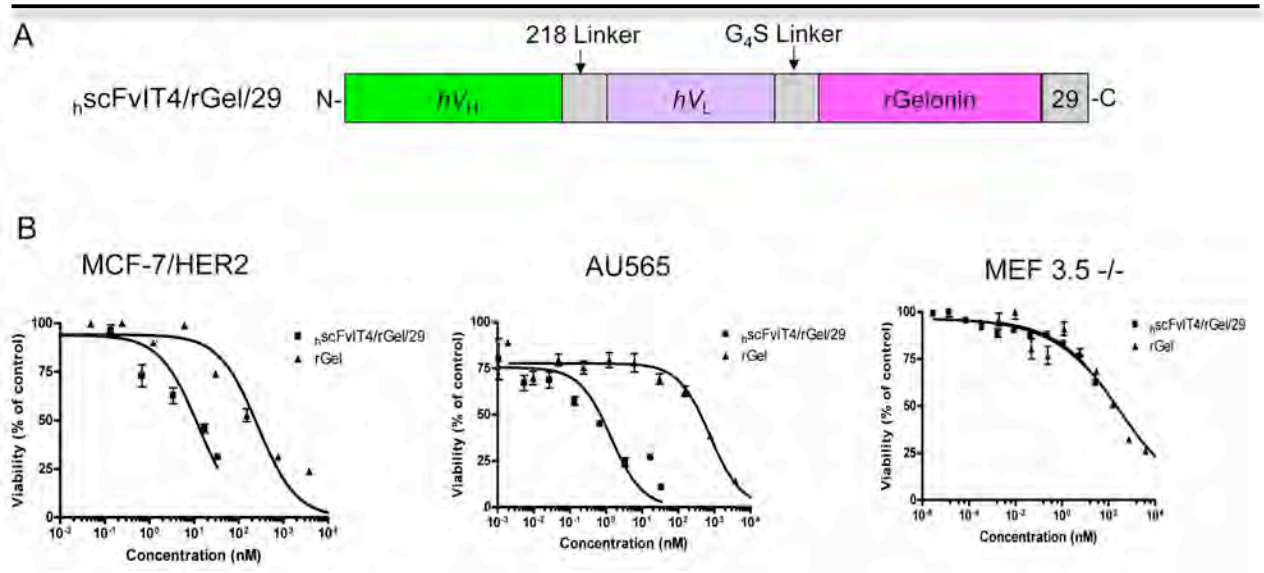

Fig. 1. The Fn14-targeted immunotoxin $_h$scFvIT4/rGel/29 has cytotoxic activity on Fn14+ but not Fn14- cells. A. Structural features of the $_h$scFvIT4/rGel/29 fusion protein. The humanized variable region heavy and light chains of the Fn14 monoclonal antibody ITEM4 are shown in green and purple, respectively. There is a flexible amino acid linker (218) to allow proper folding of the V_H and V_L domains. The G_4S linker was inserted to relieve steric stress between the V_L and the recombinant gelonin protein (magenta shading). A dimerization domain referred to as "29" is located at the carboxyl terminus. **B.** MCF-7/HER2, AU565 and Fn14-deficient fibroblasts (MEF 3.5-/-) were plated in quadruplicate in 96-well plates and allowed to adhere overnight. The cells were either left untreated or treated with various doses of purified $_h$scFvIT4/rGel/29 or rGel and incubated for 72 hr. The remaining adherent cells were stained with crystal violet and solubilized with Sorenson's buffer. Absorbance was measured at 595 nm and plotted as percentage of untreated cells.

Specific Aim 2: To determine if TWEAK-mediated cytotoxin delivery can inhibit breast tumor xenograft growth in mice.

Task Summary: Generate MDA-MB-231 breast cancer cells that express the firefly luciferase gene, perform xenograft assays in immunodeficient *nu/nu* mice to test if Fn14-targeted toxin reduces tumor growth.

Progress: We obtained MDA-MB-231-luciferase cells from a collaborator (Dr. Martin, Univ. Md. School of Med.) and confirmed that they expressed high Fn14 levels by Western blot analysis. We then determined whether the ITEM4-rGel chemical conjugate or the $_h$scFvIT4/rGel/bZ immunotoxin could inhibit the growth of MDA-MB-231 breast cancer cells *in vivo*. This immunotoxin is identical to the $_h$scFvIT4/rGel/29 protein shown in Fig. 1A except it has a different dimerization domain (named bZ). In this experiment, the saline control group tumors increased in size by 17-fold at 4 weeks. ITEM4 plus rGel, our control treatment, inhibited tumor growth by 50%, probably because ITEM4 has agonistic apoptotic activity **(Fig. 2)**. Administration

of $_h$scFvIT4/rGel/bZ or the ITEM4-rGel conjugate inhibited tumor growth by 79.5% and 99.9% at this same time point, respectively **(Fig. 2)**.

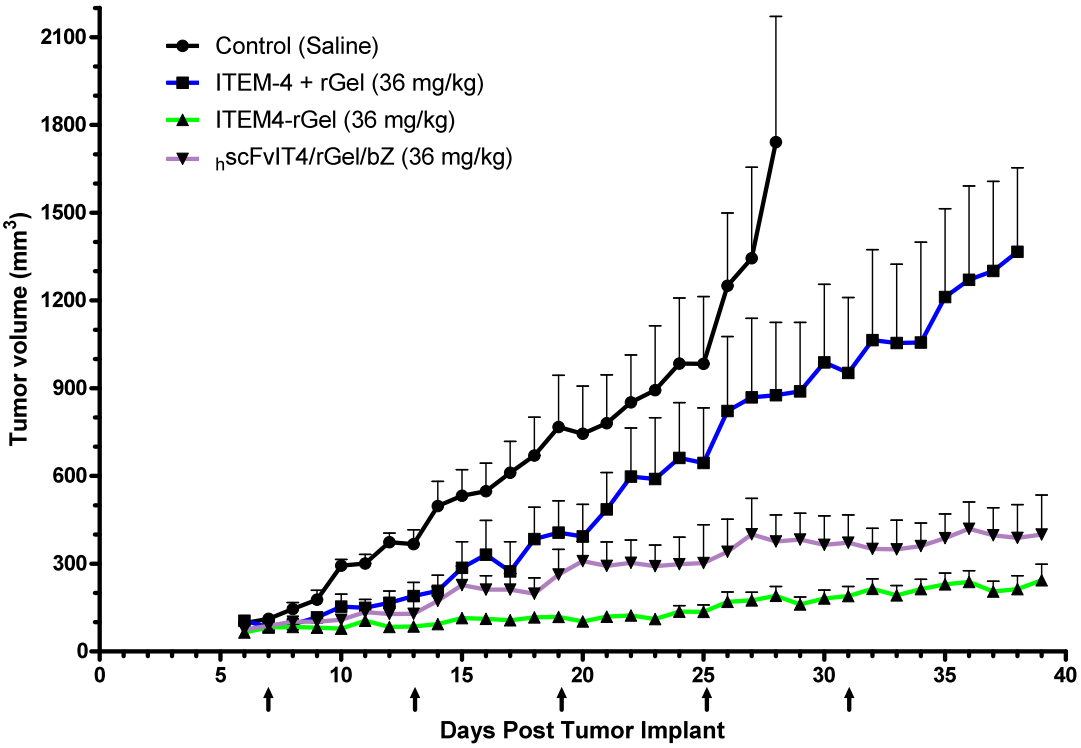

Fig. 2: ITEM4-rGel and $_h$scFvIT4/rGel/bZ administration inhibits tumor growth in the MDA-MB-231 breast tumor xenograft model. Human MDA-MB-231 breast cancer cells were injected subcutaneously into nude mice (8×10^6 cells per mouse; n = 5) and tumors were allowed to grow to approximately 100 mm^3 in volume. Mice were then injected (*i.v.* via tail vein) with either saline, ITEM-4 plus rGel, ITEM4-rGel conjugate, or $_h$scFvIT4/rGel/bZ fusion protein every 6 days as indicated with the arrows. Data is plotted as mean tumor volume (in mm^3) ± SEM.

Key Research Accomplishments:

- We found that TWEAK-PE38 toxin or TWEAK-gelonin toxin fusion proteins had some cytotoxic activity on tumor cells but these proteins were fairly difficult to purify from bacteria in an unaggregated form.
- We were able to obtain large quantities of a highly-specific Fn14 monoclonal antibody from a collaborator in Japan and prepare an ITEM4-gelonin chemical conjugate This conjugate could kill Fn14+ tumor cells *in vitro* and inhibit tumor growth *in vivo*. This work is published (4).
- We have successfully developed several single-chain immunotoxins that have cytotoxic effects on Fn14+ but not Fn14- cells. A manuscript describing this work is in preparation.

Reportable Outcomes:

- Three Abstracts (see Appendix section). Posters presented at 2010 and 2011 AACR Annual Meeting and 2011 DOD Era of Hope Breast Cancer Meeting
- One peer-reviewed publication (Reference # 4; see Appendix section)

Bibliography of all Publications and Meeting Abstracts:

Publication- Zhou, H., Marks, J.W., Hittelman**,** W.N., Yagita, H., Rosenblum, M.G. and Winkles, J.A. (2011). Development and characterization of a potent immunotoxin targeting the Fn14 receptor on solid tumor cells. Molecular Cancer Therapeutics 10:1276-1288.

Meeting Abstracts-
1. Zhou, H., Marks, J.W., Winkles, J.A., Yagita, H., and Rosenblum, M.G. (2010). Development and characterization of a potent immunotoxin targeting the Fn14 receptor on solid tumor cells. American Association for Cancer Research Annual Meeting, Washington, DC.

2. Zhou, H., Marks, J.W., Ekmekcioglu, S., Hittelman, W.N., Tran, N., Yagita, H., Winkles, J.A., and Rosenblum, M.G. (2011). Development of single chain immunotoxins targeting the fibroblast growth factor-inducible 14 (Fn14) receptor on solid tumor cells. American Association for Cancer Research Annual Meeting, Orlando, FL.

3. Zhou, H., Marks, J.W., Yagita, H., Asrani, K., Winkles, J.A., and Rosenblum, M.G. (2011). Development and characterization of Fn14 receptor-targeted immunotoxins for breast cancer therapy. DOD Breast Cancer Era of Hope Annual Meeting, Orlando, FL.

Personnel Receiving Pay from Research Effort:
Jeffrey A. Winkles
Sharron Brown

Conclusion: We conclude from this work that the best strategy for therapeutic targeting of Fn14-overexpressing breast cancers will most likely be based on an Fn14 single-chain immunotoxin molecule like that shown in Fig.1A. Future work will be to develop several additional variants of this construct, compare the activities of these molecules *in vitro,* and test their efficacy in xenograft models. This work has increased our scientific knowledge regarding the best way to target Fn14-positive breast cancer cells and in the long-term could lead to human clinical studies.

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4. Zhou, H., Marks, J.W., Hittelman**,** W.N., Yagita, H., Rosenblum, M.G. and Winkles, J.A. (2011). Development and characterization of a potent immunotoxin targeting the Fn14 receptor on solid tumor cells. Molecular Cancer Therapeutics 10:1276-1288.

Appendices: Three abstracts and one journal publication are attached.

Molecular Cancer Therapeutics

Development and Characterization of a Potent Immunoconjugate Targeting the Fn14 Receptor on Solid Tumor Cells

Hong Zhou[1], John W. Marks[1], Walter N. Hittelman[1], Hideo Yagita[3], Lawrence H. Cheung[1], Michael G. Rosenblum[1], and Jeffrey A. Winkles[2]

Abstract

TNF-like weak inducer of apoptosis (TWEAK) and fibroblast growth factor (FGF)-inducible 14 (Fn14) are a TNF superfamily ligand–receptor pair involved in many cellular processes including proliferation, migration, differentiation, inflammation, and angiogenesis. The Fn14 receptor is expressed at relatively low levels in normal tissues, but it is known to be dramatically elevated in a number of tumor types, including brain and breast tumors. Thus, it seems to be an excellent candidate for therapeutic intervention. We first analyzed Fn14 expression in human tumor cell lines. Fn14 was expressed in a variety of lines including breast, brain, bladder, skin, lung, ovarian, pancreatic, colon, prostate, and cervical cancer cell lines. We then developed an immunoconjugate containing a high-affinity anti-Fn14 monoclonal antibody (ITEM-4) conjugated to recombinant gelonin (rGel), a highly cytotoxic ribosome-inactivating *N*-glycosidase. Both ITEM-4 and the conjugate were found to bind to cells to an equivalent extent. Confocal microscopic analysis showed that ITEM4-rGel specifically and rapidly (within 2 hours) internalized into Fn14-positive T-24 bladder cancer cells but not into Fn14-deficient mouse embryonic fibroblasts. Cytotoxicity studies against 22 different tumor cell lines showed that ITEM4-rGel was highly cytotoxic to Fn14-expressing cells and was 8- to 8×10^4-fold more potent than free rGel. ITEM4-rGel was found to kill cells by inducing apoptosis with high-mobility group box 1 protein release. Finally, ITEM4-rGel immunoconjugate administration promoted long-term tumor growth suppression in nude mice bearing T-24 human bladder cancer cell xenografts. Our data support the use of an antibody–drug conjugate approach to selectively target and inhibit the growth of Fn14-expressing tumors. *Mol Cancer Ther; 10(7); 1276–88. ©2011 AACR.*

Introduction

TNF-like weak inducer of apoptosis (TWEAK) and fibroblast growth factor-inducible 14 (Fn14) are a TNF superfamily ligand–receptor pair implicated in the pathogenesis of several diseases including chronic inflammatory diseases, stroke, and cancer (1). In the cancer setting, TWEAK and Fn14 are expressed in tumor tissue and TWEAK can activate several cellular processes associated with tumor progression such as proliferation, invasion, angiogenesis, and inflammation (1, 2). However, the downstream functional consequences of TWEAK binding to Fn14 seem to depend on cellular context. For example, although TWEAK treatment of certain tumor cell lines can induce cell death (3, 4), TWEAK treatment of other tumor lines, for example, glioma cell lines, does not cause cell death but instead promotes cell migration (5) and enhanced survival following chemotherapeutic drug exposure (6, 7).

The Fn14 receptor is expressed at relatively low levels in normal tissues, but it is dramatically elevated locally in injured tissues, where it plays a role in tissue remodeling (1). In addition, the *Fn14* gene is overexpressed in multiple solid tumor types relative to matched adjacent normal tissue or normal tissue from nondiseased donors (5, 8–12). Some of these prior Fn14 overexpression reports also included data indicating that Fn14 expression levels positively correlate with tumor progression (5, 10, 11) and poor patient outcome (9). The fact that Fn14 expression is elevated in tumors as compared with normal tissue suggests that it may be a potential tumor antigen and therefore, on the basis of expression alone, a valuable therapeutic target. Recently, Culp and colleagues (8)

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Note: M.G. Rosenblum and J.A. Winkles are co-senior authors.

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reported that an anti-Fn14 monoclonal antibody (mAb) capable of inducing tumor cell apoptosis *in vitro* was efficacious in a range of tumor xenograft models, including colorectal, breast, renal, skin, and head/neck cancer models. These authors suggested that the antitumor effects occurred through both direct cell growth inhibition and antibody-dependent cellular cytotoxicity mechanisms. In consideration of these findings, this group and others (13) have proposed that therapeutic activation of the TWEAK/Fn14 pathway may represent a novel modality to inhibit tumor growth.

The use of mAbs, ligands, designed ankyrin repeat proteins (DARPins; ref. 14), and adnectins (15) for the delivery of highly cytotoxic molecules to specific target cells has gained wide acceptance and significant prominence in the field of targeted therapy. There are now several antibody–drug conjugates in clinical development and there are a number of toxin-based therapeutics under development and approved for use (16, 17). The broad tumor expression, coupled with limited normal expression, makes Fn14 an attractive candidate for a targeted therapeutic approach. We have developed an immunoconjugate designated ITEM4-rGel containing a high-affinity anti-Fn14 mAb conjugated to recombinant gelonin (rGel), a highly cytotoxic, ribosome-inactivating *N*-glycosidase. Herein, we report that this immunoconjugate can kill Fn14-positive tumor cells *in vitro* and inhibit tumor growth *in vivo*.

Materials and Methods

Materials

rGel was obtained from Goodwin Biotech. *N*-Succinimidyl-3-(2-pyridylodithio)propionate (SPDP) was from Pierce Chemicals. Sodium phosphate, sodium chloride, and EDTA were from Fisher Scientific. Sephadex G-25 gel permeation matrix (fine grade), Superose S-200 FPLC, and Blue Sepharose 6 Fast Flow were from GE Healthcare. Dithiothreitol (DTT), iodoacetamide, and dimethylformamide were from Sigma. Bradford protein assay reagent was from Bio-Rad. Acrodisc with Mustang Q membrane units were from Pall Corp. QCL-1000 endotoxin detection kits were from Lonza. Alexa Fluor 488 goat anti-rabbit IgG was purchased from Invitrogen. Fluorescein isothiocyanate (FITC)-coupled anti-rabbit IgG was from Sigma. Horseradish peroxidase–conjugated goat anti-rabbit IgG was purchased from Bio-Rad.

Cell lines and cell culture

Cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Capan-1, Capan-2, L3.6P1, AsPC-1, MIA-PaCa-2, and U-87 MG cells), DMEM/F12 (MDA-MB-231, Eb1, Calu-3, and RAW264.7 cells), RPMI 1640 (MDA-MB-435, MCF-7, BT-474, BxPc-3, NCI N-87, and Jurkat cells), McCoy's 5A (T-24, HT-29, SKOV3, ME-180, and SKBR3 cells), F12 (PC-3), or Eagle's Minimum Essential Medium (HT-1080). Fn14-deficient mouse embryonic fibroblasts (MEF3.5$^{-/-}$) were maintained in DMEM. All media contained 10% FBS. Cells were grown at 37°C with 5% CO_2 at constant humidity. Media and supplements were purchased from Invitrogen.

Construction and purification of the ITEM4-rGel conjugate

A 3-fold molar excess of the cross-linker SPDP (Pierce) was added to 2 mL of ITEM-4 (5 mg/mL in PBS) and allowed to react for 30 minutes at room temperature (RT). Excess, unreacted SPDP was removed by gel filtration using Sephadex G-25 (Amersham Biosciences) gel chromatography column. rGel (5-fold molar excess vs. ITEM-4) was reduced by adding 2 mmol/L DTT (Sigma) and stirring for 30 minutes at RT. Excess, unreacted DTT was removed by Sephadex G-25 gel chromatography. ITEM4-SPDP was slowly added to the rGel-DTT, with stirring, and the conjugation was allowed to proceed for 6 hours at 4°C under N_2 gas. Iodoacetamide (Sigma) was then added to a concentration of 2 mmol/L to block any remaining, unconjugated ITEM-4. Unconjugated rGel was removed by passage through a Superose S-75 fast protein liquid chromatography column (Amersham). The concentration of NaCl was reduced to less than 10 mmol/L by dilution, and the conjugate was applied to a Blue Sepharose (Amersham) column. Unconjugated ITEM-4 was eluted by washing with PBS (10 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.2), and the conjugate was eluted using 10 mmol/L sodium phosphate, 2 mol/L NaCl. The purified conjugate was dialyzed into PBS and concentrated using an Amicon Ultra filter (Millipore). Endotoxin levels were determined with the QCL-1000 Kit (Lonza Inc), according to the manufacturer's instructions. If the level was above 50 EU/mg protein, the sample was slowly passed through an Acrodisc Mustang Q membrane, and the endotoxin levels were then reassessed. The final conjugate preparations were then aliquoted and stored at −20°C.

Flow cytometry

To analyze Fn14 cell surface expression and cell-binding activity of ITEM4-rGel, flow cytometric analysis of cells stained with ITEM-4 or ITEM4-rGel was conducted as previously described (18). Briefly, 5×10^5 cells were incubated for 1 hour on ice with ITEM-4, ITEM4-rGel [2 μg/100 μL in 1% bovine serum albumin (BSA) in PBS], or mouse IgG2a isotype control all at the same molar concentrations. Cells were then washed twice with 0.5% BSA in PBS and incubated for an additional 30 minutes on ice with an FITC-conjugated goat anti-mouse IgG mAb. Following 2 washes, cells were fixed in 3.7% paraformaldehyde and analyzed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Surface plasmon resonance assay

Binding of ITEM-4 and ITEM4-rGel to immobilized, recombinant Fn14 extracellular domain (Cell Sciences) was measured using a BIACore 3000 instrument as

previously described (19). The binding to a blank cell (nonspecific binding) was subtracted from the sensogram.

ITEM4-rGel internalization assay

Human T-24 bladder tumor cells were plated onto polylysine-coated 16-well chamber slides (Nunc) at a density of 1×10^4 cells per well and treated with 50 nmol/L rGel for 8 hours or ITEM4-rGel for 2 or 8 hours. Proteins bound to the cell surface were removed by incubation with glycine buffer (500 mmol/L NaCl and 0.1 mol/L glycine, pH 2.5) and neutralization for 5 minutes with 0.5 mol/L Tris (pH 7.4), followed by a wash with PBS. Blocking of nonspecific protein-binding sites and immunofluorescence staining, followed by visualization using a laser scanning confocal microscope, were conducted as previously described (18).

Cytotoxicity assays

Cytotoxicity of ITEM4-rGel and rGel against various tumor cells was assessed as previously described (18). Log-phase cells were plated in 96-well flat-bottomed tissue culture plates and allowed to adhere overnight. Purified ITEM4-rGel, rGel, and/or ITEM-4 were diluted in culture medium and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 hours. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson's buffer (0.1 mol/L sodium citrate, pH 4.2, in 50% ethanol). Absorbance was measured at 595 nm. Competition cytotoxic assays were conducted by plating SKOV3 cells in 96-well plates and by preincubating the cells for 2 hours with 1 μmol/L ITEM-4 before addition of 5-fold serial dilutions of ITEM4-rGel. Results were analyzed by staining the remaining adherent cells with crystal violet as described earlier.

Apoptosis assays

Apoptosis was assessed using the Annexin V-FITC Kit (Molecular Probes, Inc.) to distinguish cells that were in the early apoptosis (Annexin V^+/PI^-) or late apoptosis/necrosis (Annexin V^+/PI^+) phase (20). Apoptosis induction through mitochondrial membrane depolarization was also investigated using the cationic dye JC-1 (JC-1 Assay Kit; MitoProbe) according to the manufacturer's instructions.

Lactate dehydrogenase release assay

The level of lactate dehydrogenase (LDH) was measured using LDH Cytotoxicity Detection Kit from Clontech Laboratories, Inc., according to the manufacturer's instructions.

Analysis of high-mobility group box 1 protein cellular release

Whole-cell lysates were resolved on 4% to 12% Criterion XT Bis-Tris gels (Bio-Rad) and transferred to a nitrocellulose membrane as previously described (18). After blocking, the membrane was incubated overnight at 4°C with anti-high-mobility group Box protein 1 (HMGB1) antibody (Santa Cruz Biotechnology; sc-74085). After incubation with peroxidase-conjugated secondary antibodies for 1 hour at RT, signals were visualized by enhanced chemiluminescence (Pierce) according to the manufacturer's instructions. Levels of HMGB1 in the culture medium were determined by Western blot analysis as previously described (21).

Xenograft tumor model and localization of ITEM4-rGel after systemic administration

Animal procedures were conducted according to a protocol approved by the AALAC-approved Animal Care and Use Facility at The University of Texas MD Anderson Cancer Center. Athymic, female nu/nu mice (8 weeks old) were obtained from Taconic Farms. Human T-24 bladder tumor cells (5×10^6) were suspended in 100 μL of PBS mixed with 100 μL Matrigel (Becton Dickinson) and then subcutaneously injected (hind flank) into the mice. Tumor volumes were determined by the formula: volume = length × width × height. When tumors reached a mean volume of ~50 to 100 mm^3, mice were randomized (5 per group) before injecting ITEM4-rGel or PBS (i.v., tail vein) every 6 days for 30 days with a total dose of 20 and 30 mg/kg. Tumor growth was measured with calipers every ~7 days. Data are presented as mean ± SEM. P values were obtained using a Student's 2-tailed t test with 95% CI for evaluation of the statistical significance compared with the controls. A value of $P < 0.05$ was considered statistically significant.

Another group of mice bearing T-24 xenograft tumors were administered ITEM4-rGel (200 μg/mouse) and PBS. Twenty-four hours later, animals were euthanized and tumor tissue was removed, snap-frozen, and sectioned. To examine the presence of ITEM4-rGel, the sections were dried and then fixed in 3.7% formaldehyde (Sigma) for 20 minutes at RT followed by a brief rinse with PBS. Cells were then permeabilized for 10 minutes in PBS containing 0.2% Triton X-100, washed 3 times with PBS, and blocked with PBS containing 3% BSA for 1 hour at RT. Fixed cells were incubated with rabbit anti-rGel antibody (22) for 2 hours at RT. The slides were washed with PBS and then incubated with anti-rabbit IgG-FITC–conjugated antibody. Cell nuclei were counterstained by exposure to propidium iodide (PI; 1 μg/mL) for 1 hour at RT. After a final wash step, the slides were mounted and analyzed under a fluorescence microscope.

Terminal deoxynucleotidyl transferase–mediated nick end labeling assay to detect apoptosis

The T-24 tumor–frozen sections were stained by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) using an *in situ* cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Samples were analyzed under a Nikon Eclipse TS100 fluorescent microscope,

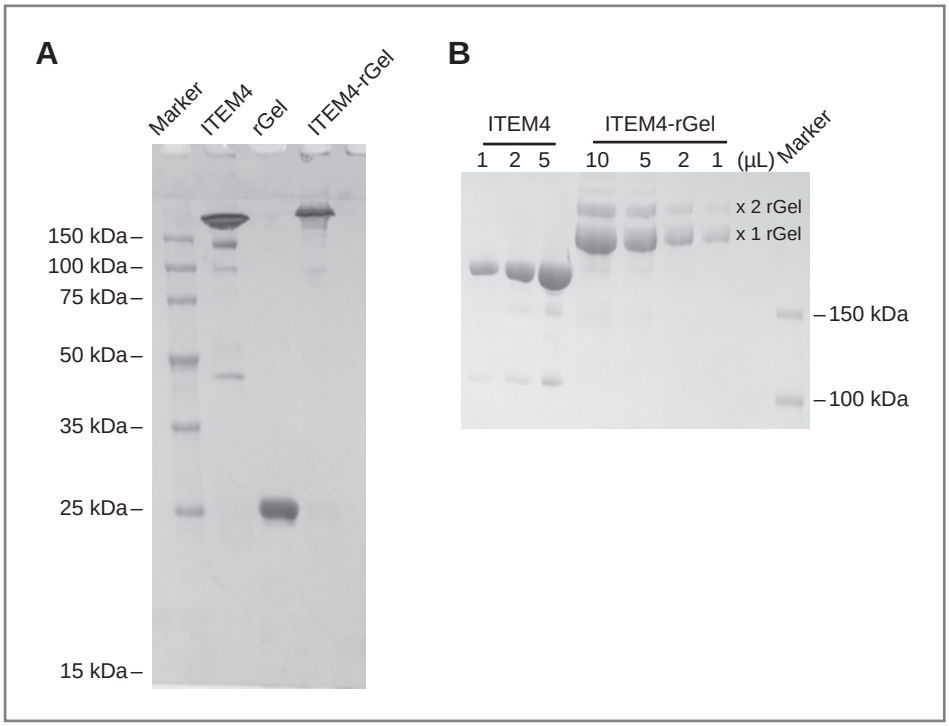

Figure 1. ITEM4-rGel conjugate preparation and purification. A, SDS-PAGE analysis of the purified ITEM-4, rGel, and ITEM4-rGel immunoconjugate on 10% nonreduced gel. B, SDS-PAGE analysis of ITEM4-rGel with different loading volumes on 6% nonreduced gel. The resultant ITEM4-rGel was composed of antibody + 1 rGel (predominant) and antibody + 2 rGel (minor) species and was essentially free of contaminating rGel or unreacted ITEM-4 antibody.

and photographs were taken with a scope-mounted Nikon digital camera.

Results

Preparation of ITEM4-rGel immunoconjugate

We used the high-affinity murine anti-Fn14 mAb ITEM-4 (3) to generate a chemical conjugate with recombinant rGel toxin (designated ITEM4-rGel), using the heterobifunctional cross-linker SPDP as described in Materials and Methods. The ITEM4-rGel conjugate was purified and the final product was found to contain no contaminating free antibody or rGel as shown in Fig. 1A. Analysis of the preparation confirmed that the final material contained both antibody + 1 rGel (major) and antibody + 2 rGel (minor) species (Fig. 1B).

The TWEAK receptor Fn14 is overexpressed in multiple tumor cell lines

We next examined Fn14 expression in a panel of normal and tumor cell lines by both Western blot analysis and flow cytometry. Fn14 expression was detected by Western blotting in 17 of 21 tumor cell lines tested (Fig. 2A). A mouse macrophage cell line (RAW264.7) and an embryonic fibroblast cell line generated from Fn14-deficient mice did not express Fn14. A similar pattern of Fn14 expression was found by flow cytometry with the ITEM-4 mAb (Fig. 2B and Table 1). These results show that a large number of tumor cell lines representing multiple tumor types constitutively express the TWEAK receptor Fn14.

ITEM4-rGel specifically binds to the Fn14 receptor and is internalized into Fn14-positive T-24 cells

To examine whether modification of the ITEM-4 antibody through the conjugation process affected the binding to Fn14, ITEM-4 and ITEM4-rGel were tested for binding to both recombinant Fn14 and Fn14-expressing cells. ITEM-4 and ITEM4-rGel binding to recombinant Fn14 extracellular domain was determined by surface plasmon resonance analysis by using a BIAcore instrument. We found that ITEM-4 and ITEM4-rGel bound to Fn14 with similar equilibrium dissociation constants (K_d) of ~1.1 and 0.7 nmol/L, respectively (Fig. 3A). Purified rGel did not bind to Fn14 in this assay. We could find no differences in the flow cytometric binding curves for ITEM-4 (Fig. 2B) or ITEM4-rGel (Fig. 3B) by using Fn14-expressing cells (MDA-MB-231, T-24, and HT-29) as targets. Although it is known that ITEM-4 recognizes the murine Fn14 receptor (23), neither antibody nor immunoconjugate was found to bind to Fn14-deficient MEF cells as determined by flow cytometry (Figs. 2B and 3B). These data show that the binding affinity and the selectivity of the native ITEM-4 antibody seemed to be unaffected by conjugation to the rGel toxin.

We next examined the ability of ITEM4-rGel to internalize and deliver rGel to the cytoplasm of Fn14-expressing T-24 tumor cells. Immunofluoresence studies showed that exposure of cells to ITEM4-rGel resulted in efficient, rapid internalization of the rGel component to the cytoplasm (Fig. 3C). It is important to note that in processing treated cells for internalization analysis, the use of brief acid exposure to remove surface-bound immunoconjugate permits preferential detection of internalized

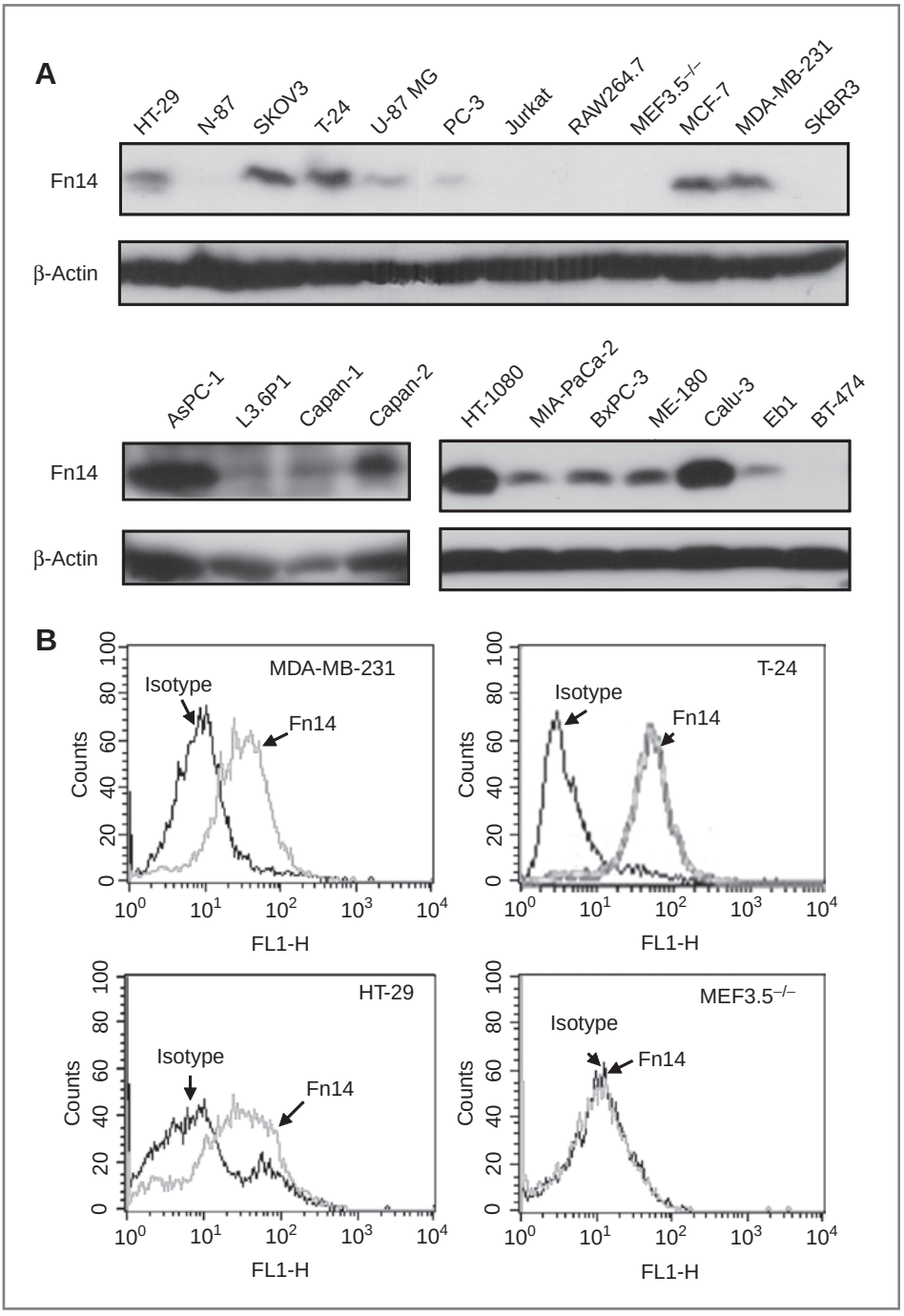

Figure 2. Fn14 receptor expression in various tumor cell lines. A, expression of Fn14 protein by 21 different human tumor cell lines was assayed by Western blotting by using anti-Fn14 mAb ITEM-4. β-Actin was used as a control for protein loading. B, Fn14 expression analysis by flow cytometry. MDA-MB-231, T-24, HT-29, and MEF3.5$^{-/-}$ cells were incubated with ITEM-4 followed by FITC-conjugated goat anti-mouse IgG antibody and analyzed with a FACSCalibur flow cytometer. Black curve, isotype control (cells + mouse IgG2a + FITC-Ab); gray curve, Fn14 (cells + ITEM-4 + FITC-Ab).

conjugate. When these cells were treated with rGel alone, no internalization was detected (Fig. 3C). In addition, ITEM4-rGel internalization was not observed when the conjugate was added to Fn14-deficient mouse embryonic fibroblasts (data not shown).

ITEM4-rGel is highly cytotoxic to Fn14-expressing tumor cells

We examined the cytotoxic effects of ITEM4-rGel on cell lines expressing various levels of Fn14 to determine the general sensitivity of Fn14-expressing tumor cells and to correlate the cytotoxic effects of the immunoconjugate to Fn14 expression levels. The targeting index (the ratio of IC_{50} for rGel vs. the IC_{50} for ITEM4-rGel) was calculated for each cell line. This ratio represents the ability of the ITEM-4 component of the ITEM4-rGel immunoconjugate to mediate delivery of the rGel toxin component to the target cell cytoplasm and normalizes for the inherent cellular sensitivity to the rGel toxin. As shown in Table 1, the highest targeting index was found

Table 1. Fn14 status and cytotoxic effect of ITEM4-rGel on various normal and tumor cell lines

Cell lines	Tumor types	Fn14 receptor	rGel IC_{50} (nmol/L)	ITEM4-rGel IC_{50} (nmol/L)	Targeting index[a]
Eb1	Breast	+	835	0.01	83,500
MDA-MB-231	Breast	+++	717.7	0.08	8,241
MCF-7	Breast	+++	237.4	0.03	7,913
SKBR3	Breast	++	2,712	10.6	255
BT-474	Breast	–	217	None	
Capan-2	Pancreatic	++++	2,270	0.1	22,700
MIA-PaCa-2	Pancreatic	++	638.7	0.1	6,387
AsPC-1	Pancreatic	++++	3,980	0.8	4,574
BxPC-3	Pancreatic	++	295.6	0.2	1,615
L3.6P1	Pancreatic	++	230	0.6	460
Capan-1	Pancreatic	++	8,750	92.4	92
MDA-MB-435	Melanoma	++++	218.5	0.005	43,700
HT-1080	Fibrosarcoma	++++	286.4	0.02	14,320
SKOV3	Ovarian	+++	1,376	0.4	3,822
U-87 MG	Glioblastoma	+	13.2	0.01	1,321
PC-3	Prostate	+	117.1	0.09	1,301
T-24	Bladder	+++	72.8	0.06	1,213
HT-29	Colon	+++	974.9	7.3	132
ME-180	Cervix	+++	1,222	25.1	48
NCI N-87	Gastric	+	1,085	78.3	13
Calu-3	Lung	++++	121.7	13.8	8
Jurkat	T lymphoma	–	899.5	None	–
RAW264.7	Mouse macrophage	–	172.7	139.9	1
MEF3.5$^{-/-}$	Mouse embryonic fibroblast	–	652.7	514.5	1

NOTE: Fn14 expression is based on the percentage of cells stained with ITEM-4 and detected by flow cytometry: –, negative; +, <10% positive; 11%–25% positive; ++, 26%–50% positive; +++, 51%–75% positive; ++++, >75% positive.
[a]Targeting index is defined as: (IC_{50} of rGel)/(IC_{50} of ITEM4-rGel).

in breast, pancreatic, and melanoma tumor lines. The breast tumor cell line Eb1 was found to be the most sensitive to ITEM4-rGel (targeting index = 83,500). A number of cell lines that express low levels of Fn14 (Eb1, U-87 MG, and PC-3) were nevertheless sensitive to ITEM4-rGel treatment. Overall, ITEM4-rGel was shown to be 8- to 8 $\times 10^4$-fold more potent than free rGel. Cell lines that were negative for Fn14 expression were not sensitive to the conjugate.

The cytotoxicity profile of ITEM4-rGel, rGel, and ITEM-4 following addition to MDA-MB-231, HT-29, T-24, BxPC-3, MDA-MB-435, and Fn14-deficient MEF cells in culture is shown in Fig. 4A. ITEM4-rGel could specifically kill Fn14-expressing cells in a dose-dependent manner, whereas native ITEM-4 antibody alone had no effect at doses of up to 1 μmol/L. In comparison, the cytotoxic effects of ITEM4-rGel on nontarget MEF cells were similar to the free rGel with a targeting index of 1.

To confirm that Fn14 binding was required for cytotoxicity of the conjugate, we first preincubated SKOV3 cells with 1 μmol/L ITEM-4 for 2 hours before addition of various amounts of ITEM4-rGel. As shown in Fig. 4B, ITEM4-rGel was cytotoxic to SKOV3 cells with an IC_{50} value of 0.4 nmol/L. Preincubation with 1 μmol/L ITEM-4 for 2 hours completely abrogated ITEM4-rGel–induced cytotoxicity, thereby confirming that initial binding of ITEM4-rGel to the Fn14 receptor is required to initiate the cytotoxic effects of the conjugate.

We next examined the minimal contact time required for the ITEM4-rGel conjugate to generate the optimum biological effect. We exposed MDA-MB-231 and T-24 cells to various ITEM4-rGel concentrations for various times ranging from 1 to 72 hours. As summarized in Table 2, the lowest IC_{50} doses were observed after 24 hours of exposure and there was no appreciable increase in sensitivity of cells for longer exposure times.

ITEM4-rGel treatment induces apoptosis characterized by HMGB1 release

The mechanisms responsible for the cytotoxicity of ITEM4-rGel were investigated using human T-24 bladder carcinoma cells. These cells were treated with 1 nmol/L rGel, ITEM-4, or ITEM4-rGel for 72 hours and then subjected to Annexin V and PI staining. Treatment with ITEM4-rGel resulted in a significant increase in Annexin V–positive cells, suggesting that the conjugate promotes

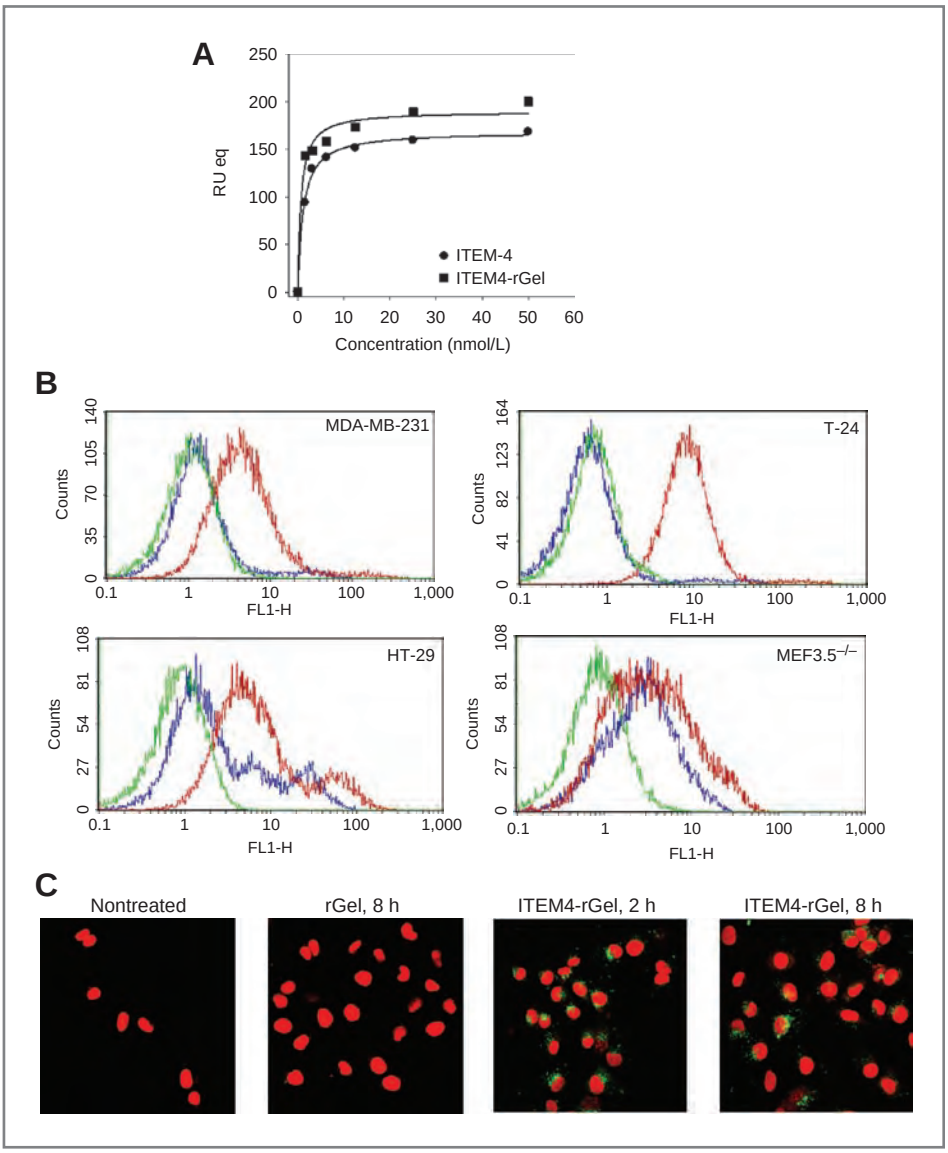

Figure 3. ITEM4-rGel specifically binds and internalizes into Fn14-expressing tumor cells. A, surface plasmon resonance analysis of ITEM-4 and ITEM4-rGel binding to immobilized recombinant Fn14 extracellular domain. RU, response unit or resonance unit. B, MDA-MB-231, T-24, HT-29, and MEF3.5$^{-/-}$ cells were incubated with ITEM4-rGel followed by FITC-conjugated goat anti-mouse IgG antibody and analyzed with a FACSCalibur flow cytometer. Green curve, untreated cells; blue curve, isotype control (cells + mouse IgG + FITC-Ab); and red curve, ITEM4-rGel (cells + ITEM4-rGel + FITC-Ab). C, T-24 cells were either left untreated or treated with either 50 nmol/L ITEM4-rGel for 2 and 8 hours or rGel for 8 hours. The cells were fixed, acid washed to remove surface-bound material, permeabilized, and immunostained for the presence of rGel by using a rabbit anti-rGel antibody (green). The cells were counterstained with PI (red) to identify nuclei and visualized using a laser scanning confocal microscope.

target cell apoptosis (Fig. 5A). We also showed that exposure of T-24 cells to ITEM4-rGel, but not ITEM-4 or rGel, resulted in mitochondrial membrane depolarization, using the mitochondrial dye JC-1 (Fig. 5B). This finding is also consistent with an apoptotic cell death mechanism.

It has been reported that most forms of tumor cell death result in release of the RAGE ligand HMGB1 (21). Therefore, we tested whether HMGB1 would be released from tumor cells following treatment with ITEM4-rGel. We treated T-24 cells with rGel, ITEM-4, or ITEM4-rGel for 24 hours and assessed HMGB1 levels in cell lysates and conditioned media by Western blot analysis. We observed that treatment of cells with both ITEM-4 and ITEM4-rGel resulted in HMGB1 release (Fig. 5C), which usually occurs in cells that are undergoing classical necrotic cell death marked by abrupt membrane lysis and the release of soluble proteins (24). To evaluate the release of another soluble protein, we measured LDH release in treated T-24 cells and found that neither ITEM-4 nor ITEM4-rGel treatment resulted in LDH release (Fig. 5D). Treatment of cells with the ITEM-4 antibody did not have demonstrable cytotoxic effects, nor did it cause measurable apoptotic damage. These data indicate that HMGB1 release from ITEM4-treated cells did not seem to be associated with necrosis but instead may represent selective release of the HMGB1 protein from cells.

ITEM4-rGel localizes in T-24 cell xenograft tumors after intravenous administration

We next investigated whether the ITEM4-rGel conjugate was delivered to xenograft tumors following intravenous injection. The ITEM4-rGel conjugate or PBS was

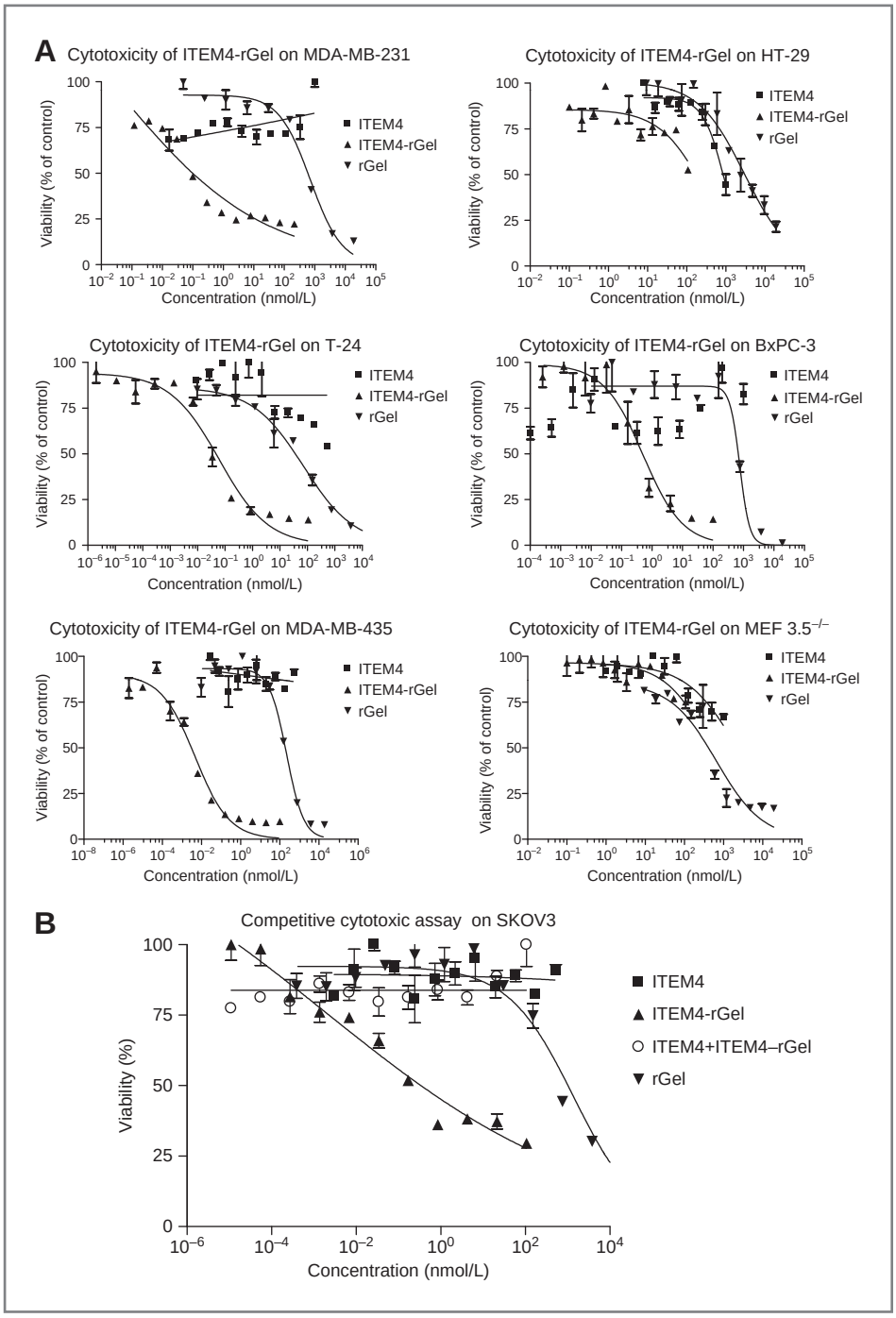

Figure 4. Cytotoxicity of ITEM4-rGel when added to different tumor cell lines. A, different concentrations of ITEM-4, rGel, and ITEM4-rGel were added to various tumor cell lines (MDA-MB-231, HT-29, T-24, BxPC-3, and MDA-MB-435) and Fn14-deficient mouse embryonic fibroblasts (MEF3.5$^{-/-}$), and cytotoxicity was measured as described in Materials and Methods. B, SKOV3 cells were either left untreated or incubated with different concentrations of ITEM-4, ITEM-4-rGel, or rGel for 72 hours or pretreated with 1 μmol/L ITEM-4 for 2 hours and then coincubated with different concentrations of ITEM4-rGel for another 72 hours. Cytotoxic effects were assessed as above.

injected into mice bearing T-24 bladder tumors and the tumors were harvested 24 hours later. Tumor sections were made and assayed for the presence of ITEM4-rGel by immunofluorescence by using an anti-rGel antibody. As shown in Fig. 6A, tumors obtained from the ITEM4-rGel–injected mice showed staining with the rGel antibody whereas there was no staining observed in the saline-treated group. In general, the intratumoral localization of the ITEM4-rGel conjugate (green) appeared to be relatively uniform throughout the tumor sections stained, although there were a few areas of intense staining noted.

Inhibition of tumor growth *in vivo* by ITEM4-rGel

We then determined whether ITEM4-rGel administration could inhibit the growth of T-24 bladder tumor xenografts growing subcutaneously (flank) in nude mice. T-24 cells were injected into mice and when tumors

Table 2. *In vitro* cytotoxicity of ITEM4-rGel and exposure duration

Drug exposure time (h)	IC_{50} (nmol/L)	
	T-24	MDA-MB-231
1	1.4	0.4
3	2.4	0.7
8	0.2	0.3
24	0.03	0.01
48	0.04	0.02
72	0.07	0.06

reached a mean volume of ~50 to 100 mm^3 mice were randomized (5 per group) before injecting either ITEM4-rGel or PBS (i.v., tail vein) every 6 days for 30 days with a total dose of 20 or 30 mg/kg. As shown in Fig. 6B, tumor volumes in the saline-treated control mice increased ~16-fold (from ~50 mm^3 to a mean of 800 mm^3) over the 125-day course of the experiment. In contrast, tumors from the ITEM4-rGel–treated mice increased ~3.6-fold (from ~50 mm^3 to a mean of 182 mm^3) and showed no change in tumor size over the same period for 20 and 30 mg/kg dosage groups, respectively. There were no animal deaths in the studies, and mouse body weights showed no significant change in any of the treated or control groups over the duration of the experiment (data not shown). We next examined tumors from the control and treated groups. Histological (hematoxyline–eosin) stain of tumors from the control and treated groups at 125 days showed viable tumor cells in all groups (data not shown). However, as shown in Fig. 6C, there was a significant increase in the number of apoptotic cells (as assessed by TUNEL staining) in tumors from mice treated with ITEM4-rGel.

Discussion

Various TNF and TNF receptor (TNFR) superfamily members have been identified as being essential elements in tumor growth and development (25–27). In addition, they have also been implicated in the response and resistance of tumor cells to therapeutic agents (28, 29). For many of these reasons, TNF family member ligands and their cognate receptors seem to be attractive targets for intervention and for the development of targeted therapeutics for the treatment of cancer and immunologic diseases (28, 30, 31). One particular TNF:TNFR pair, TWEAK and Fn14, has been implicated in several important processes associated with tumor growth and metastasis (5–13, 32, 33), and a recent report has shown that administration of an anti-Fn14 mAb can inhibit tumor growth in xenograft assays (8).

The use of antibodies to interfere with growth factors and their receptors present on tumors has gained wide acceptance as a therapeutic strategy (34–36). Targeting the HER1, HER2, and VEGF pathways with antibodies is now considered an essential component in the clinical therapeutic management of a variety of tumor types (37). There are also numerous groups developing antibody conjugates for the directed delivery of highly cytotoxic payloads such as small molecules—auristatin, caliecheamycin, or maytansinoids (38–41), protein toxins, that is, RTA, PE, and gelonin (22, 42, 43), or other cytotoxic human enzymes such as serine proteases (44). The current study shows a unique Fn14 receptor–targeted antibody–toxin conjugate designated ITEM4-rGel that is shown to be capable of specifically delivering rGel to Fn14-expressing tumor cells *in vitro* and *in vivo*. As described previously, activation of TWEAK signaling by either ligands or antibodies has the potential to elicit progrowth effects in addition to defined cytotoxic properties. The use of cytotoxic conjugates targeting Fn14 has the potential to augment the cytotoxic effects of pathway activation while eliminating the growth stimulatory properties.

The antibody ITEM-4 and its corresponding immunoconjugate ITEM4-rGel was shown to selectively bind the Fn14 receptor on tumor cells. The cytotoxicity of ITEM4-rGel on Fn14-expressing tumor cells was investigated extensively *in vitro*. ITEM4-rGel was found to be selectively toxic to a wide range of human tumor cell lines expressing the Fn14 receptor (Table 1). Cells overexpressing Fn14 were highly sensitive to the ITEM4-rGel, whereas cells expressing no Fn14 were no more sensitive to the conjugate than they were to free rGel. Surprisingly, some cell lines expressing high (Calu-3) or intermediate (ME-180 and HT-29) levels of Fn14 showed relatively low levels of sensitivity to the construct. Alternatively, Eb1 breast tumor cells expressing low levels of Fn14 showed extreme sensitivity to the construct. The observed differences in cellular response to the conjugate may be the result of differences in cellular protein synthesis rates, receptor recycling, immunotoxin internalization efficiency (45, 46), or intracellular trafficking and release from endosomal compartments (47). All of these factors may play a role in determining the ultimate response of cells to the ITEM4-rGel construct.

Our previous studies with rGel-based constructs showed that some constructs induced apoptosis (48), but some did not, and this seemed to be highly dependent on the cell type under investigation (49). We found that the ITEM4-rGel induced cell death with clear mitochondrial dependent apoptosis. We also showed that when T-24 cells were treated with either ITEM-4 alone or ITEM4-rGel, the protein HMGB1 was released into the media. The effect of unconjugated ITEM-4 on HMGB1 release was unexpected, as ITEM-4 showed no effect on the growth of these cells in culture. The effect of ITEM-4 on HMGB1 release by tumor cells has not been reported previously, but a study by Kalinina and colleagues (50) reported that treatment with the Fn14 ligand TWEAK caused an upregulation of HMGB1 synthesis and

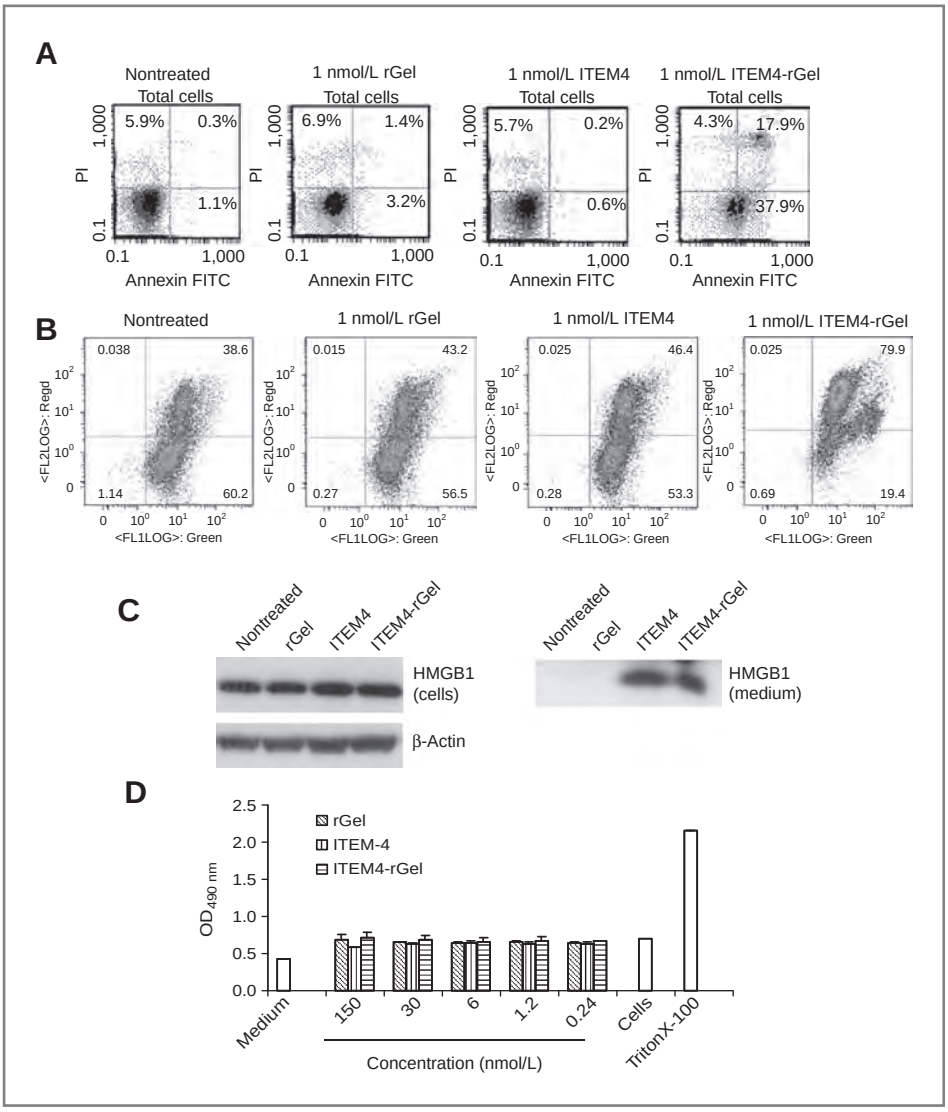

Figure 5. Analysis of ITEM4-rGel-induced cell death. A, T-24 cells were either left untreated or treated with rGel, ITEM-4, or ITEM4-rGel for 72 hours and then stained with Alexa Fluor 488 Annexin V and PI, followed by flow cytometric analysis. Numbers in the quadrants, percentage of cells in each category. B, T-24 cells were either left untreated or treated with rGel, ITEM-4, or ITEM4-rGel for 72 hours and then mitochondrial membrane depolarization was assayed by JC-1 staining followed by flow cytometric analysis. Numbers in the quadrants, percentage of cells of each category. C, Western blot analysis of cell extract and conditioned media for HMGB1 protein after treatment of T-24 cells with rGel, ITEM-4, or ITEM4-rGel for 24 hours. D, T-24 cells were treated with different concentrations of rGel, ITEM-4, and ITEM4-rGel for 24 hours and LDH release was measured. OD, optical density. Data shown are mean ± SD from 3 replicates. Values for medium alone represent baseline LDH for culture media containing 1% FBS. Values for cells in media represent untreated cells. Values for Triton X-100 represent the maximal release of LDH from the cells after detergent treatment.

secretion by normal cells as part of the inflammatory process. It is possible that ITEM-4 agonistic effect on the Fn14 receptor could cause release of HMGB1 in certain cell types.

Fn14 is gathering much attention at this time because of its strong overexpression in many of the most prevalent and deadly solid tumor types. We used the T-24 bladder model because the *in vitro* response to the cytotoxic effects of the conjugate and the relative expression levels of the Fn14 receptor (via Western blot) seemed to be intermediate in the range of cell lines tested. The results of our studies using the T-24 xenograft model show that an immunoconjugate targeting Fn14 can suppress tumor growth. The maximum tolerated dose (MTD) for ITEM4-rGel with this schedule was determined to be 65 mg/kg (data not shown) and we selected 20 and 30 mg/kg total dosages (30% and 50% of the MTD, respectively) for these initial studies. The *in vivo* data show that effective tumor cell killing (by induction of apoptosis as assayed by TUNEL assay) can be achieved with ITEM4-rGel at these doses without any observable side effects in mice suggest that targeting of the conjugate to normal mouse tissues is minimal. The immunohistochemical studies showed that ITEM4-rGel uniformly distributed in tumor xenografts after intravenous administration, although additional studies are ongoing to examine the pharmacokinetics and the relative uptake kinetics of ITEM4-rGel into tumor compared with normal organs. The results from these studies may define a new schedule of administration to optimize tumor uptake of the immunoconjugate.

In summary, this study shows a proof of concept that Fn14 seems to have an excellent potential for targeted therapy approaches and ITEM-4–based therapeutic agents seem to warrant further development. Although therapeutic studies with ITEM4-rGel show *in vivo* efficacy, generating a human or humanized version of

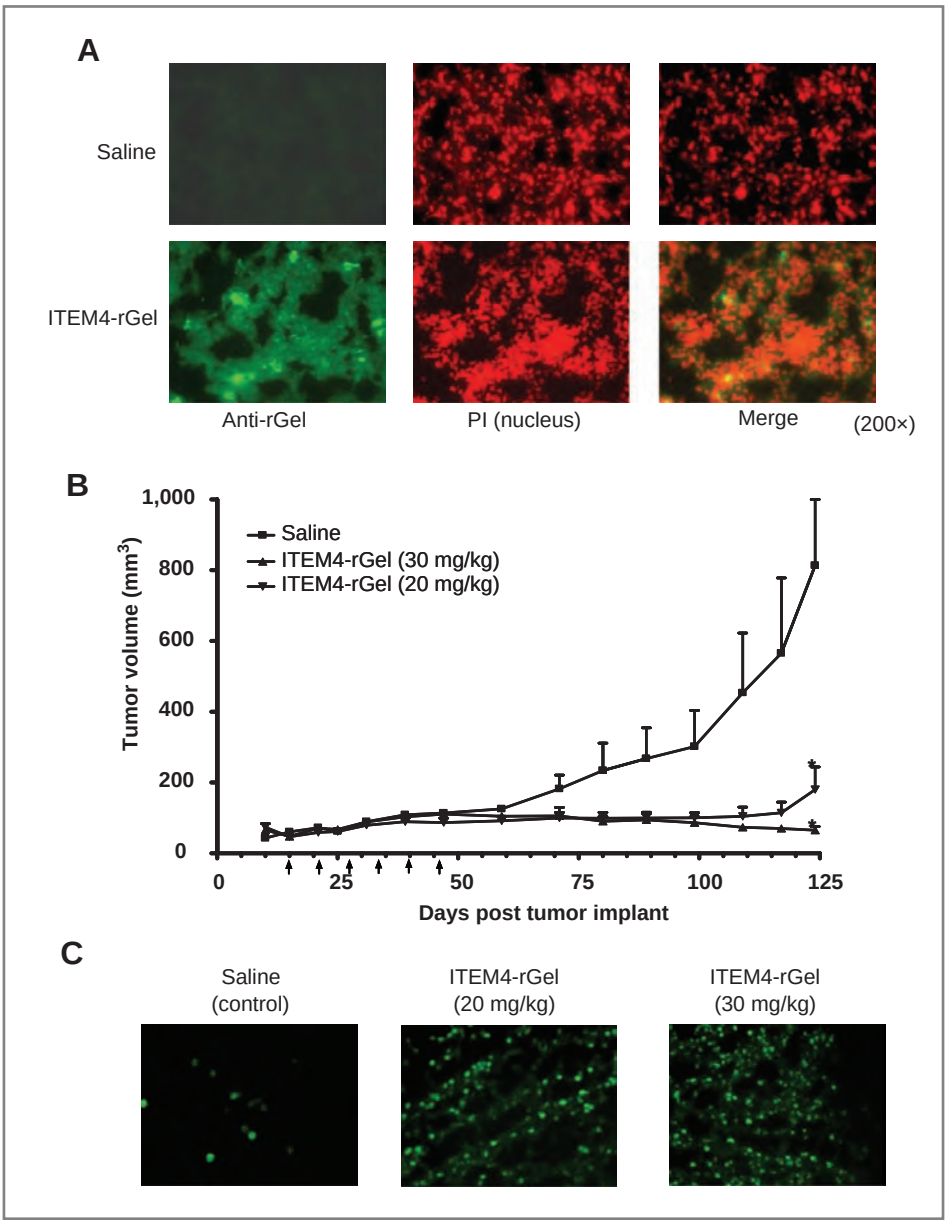

Figure 6. ITEM-4-rGel administered to mice localizes in tumor tissue and inhibits tumor growth. A, ITEM4-rGel was administered (i.v.) to mice bearing T-24 bladder carcinoma xenograft tumors. One day later, animals were sacrificed and tumor tissues were removed, fixed, sectioned, and assayed for the presence of ITEM4-rGel by immunofluorescence by using a rabbit anti-rGel antibody (green). The cells were counterstained with PI (red) to identify nuclei and visualized using a Nikon Eclipse TS 100 fluorescent microscope. B, T-24 cells were injected subcutaneously into mice and tumors were allowed to grow to 50 to 100 mm^3 in volume. Mice ($n = 5$) were injected (i.v., tail vein) with either saline (control) or the ITEM4-rGel conjugate 6 times at 20 or 30 mg/kg total dose. Data represent mean tumor volumes $\pm$ SEM; *, significant difference ($P < 0.05$) when comparing ITEM4-rGel treated mice versus the control group. C, apoptosis detection in tumor tissue by TUNEL assay. Mice bearing T-24 xenograft tumors were administered (i.v.) with either saline (control) or the ITEM4-rGel (20 and 30 mg/kg). Tumor tissue sections were stained by TUNEL and analyzed under a Nikon Eclipse TS 100 fluorescent microscope.

ITEM-4 is essential for long-term clinical administration. The immunogenicity of the rGel component of the conjugate is of potential concern, although clinical studies of an immunoconjugate with the anti-CD33 antibody HuM195 have shown limited antigenicity of the rGel component even with repeated administration (Cortes and colleagues, unpublished data). Studies of anti-Fn14 constructs containing second-generation payloads such as deimmunized rGel toxin (designated drGel) or fully human granzyme B as a replacement for full-length rGel are in progress.

Disclosure of Potential Conflicts of Interest

M.G. Rosenblum has patents.

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AACR 2010 Meeting Abstract

Development and characterization of a potent immunotoxin targeting the Fn14 receptor on solid tumor cells

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TNF-like weak inducer of apoptosis (TWEAK) and FGF-inducible 14 (Fn14) are a TNF superfamily ligand-receptor pair involved in inflammation, oncogenesis, tumor invasion, migration, survival and resistance to chemotherapy. The Fn14 receptor is expressed at relatively low levels in normal tissues, but is known to be dramatically elevated in a number of tumor types, including brain and breast tumors. Thus, the TWEAK/Fn14 axis appears to be an excellent candidate for therapeutic intervention. We have developed an immunoconjugate designated ITEM4-rGel containing a high-affinity anti-Fn14 monoclonal antibody conjugated to recombinant gelonin (rGel), a highly cytotoxic, ribosome-inactivating n-glycosidase. The ITEM4-rGel conjugate was generated and purified and contained no contaminating free antibody or rGel. The final material contained both antibody + 1 rGel (major) and antibody + 2 rGel (minor) species. We analyzed Fn14 expression in human tumor cell lines using flow cytometry and Western blot analysis. Fn14 was expressed in a variety of tumor lines including breast, brain, bladder, skin, lung, ovarian, pancreatic, colon, prostate, and cervical tumor cell lines. Both ITEM4 and ITEM4-rGel were found to bind to cells to an equivalent extent. Confocal immunofluoresence studies showed that ITEM4-rGel specifically and rapidly (within 2 hrs) internalized into MDA-MB-231 breast cancer cells and T-24 bladder cancer cells but not into Fn14-deficient mouse embryonic fibroblasts. Cytotoxicity studies against 22 different tumor cell lines showed that ITEM4-rGel was highly cytotoxic to Fn14-expressing cells (IC_{50} ranged from 0.8 pM-25 nM) and was 50 to 0.5 $x10^6$ fold more potent than free rGel. Minimum contact time studies showed that as little as 12 hr exposure achieved maximal cytotoxic effect. Mechanistic studies showed that ITEM4-rGel induced HMGB1 release following treatment of MDA-MB-231, T-24, AAB 527, and BxPC-3 cells. In addition, target cells showed induction of apoptosis, as measured by Annexin V staining and caspase-3 cleavage. ITEM4-rGel treatment also induced the non-canonical NF-κB pathway, up-regulated the tumor suppressor protein p53 and down-regulated survivin expression. Preliminary mouse xenograft tumor model studies are ongoing. These data suggest that the ITEM4-rGel construct may warrant further development as a novel therapeutic agent against a broad range of solid tumor types. Research conducted, in part, by the Clayton Foundation for Research (MGR), and supported by NIH grant NS55126 (JAW) and DOD Breast Cancer Concept Award BC086135 (JAW).

AACR 2011 Meeting Abstract

Development of Single Chain Immunotoxins Targeting the Fibroblast Growth Factor-inducible 14 (Fn14) Receptor on Solid Tumor Cells

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Previous studies have indicated that Fn14, the cell surface receptor for the cytokine TWEAK, is over-expressed in multiple human solid tumor types, including brain, breast, pancreatic, esophageal, colon and lung cancers. Using a melanoma tumor array, we recently found that Fn14 expression is elevated in 177/190 (93%) primary melanoma specimens and in 87/150 (58%) melanoma metastases specimens. This suggests that Fn14 may be a novel therapeutic target in this disease setting as well. We developed an immunoconjugate designated ITEM4-rGel composed of a high-affinity anti-Fn14 monoclonal antibody (ITEM-4) conjugated to recombinant gelonin (rGel), a highly cytotoxic, ribosome-inactivating n-glycosidase. To develop Fn14-targeted immunotoxins more suitable for clinical use, a humanized single-chain version of ITEM-4 (designated $_{h}$scFvIT4) specific for Fn14 was successfully produced and characterized. BiaCore analysis of ITEM-4, ITEM4-rGel and $_{h}$scFvIT4 binding to the Fn14 extracellular domain indicated that these proteins bound to Fn14 with similar affinity, with K_ds of ~ 1.1 nM, ~ 0.7 nM, and ~ 6.2 nM, respectively. Using $_{h}$scFvIT4 and rGel toxin, we next engineered a bivalent immunotoxin ($_{h}$scFvIT4/rGel/29) by adding a COOH-terminal dimerization domain. Cytotoxicity studies showed that ITEM4-rGel and its humanized counterpart, $_{h}$scFvIT4/rGel/29, were highly cytotoxic to Fn14-expressing cells (IC_{50} ranged from 0.8 pM-25 nM) and were 50 to 0.5 x10^6 fold more potent than free rGel. Minimum contact time studies showed that as little as 8 hr exposure achieved maximal cytotoxic effect. Both ITEM4-rGel and $_{h}$scFvIT4/rGel/29 were shown to specifically bind to Fn14-expressing tumor cells as analyzed by ELISA. Confocal immunofluoresence studies showed that ITEM4-rGel and $_{h}$scFvIT4/rGel/29 specifically and rapidly (within 2 hrs) internalized into MDA-MB-435 melanoma cells but not into Fn14-deficient mouse embryonic fibroblasts. Mechanistic studies showed that ITEM4-rGel induced apoptosis in antigen-positive T24 human bladder carcinoma cells as measured by Annexin V staining. Treatment of MDA-MB-435 cells with $_{h}$scFvIT4/rGel/29 induced the non-canonical NF-κB pathway and down-regulated survivin expression. Preliminary mouse xenograft tumor model studies are ongoing. We propose that the $_{h}$scFvIT4/rGel/29 construct may warrant further development as a novel therapeutic agent against a broad range of Fn14-positive solid tumor types.

Research conducted, in part, by the Clayton Foundation for Research (MGR), and supported by NIH grant NS55126 (JAW) and DOD Breast Cancer Concept Award BC086135 (JAW).

DOD Era of Hope Meeting 2011 Abstract

DEVELOPMENT AND CHARACTERIZATION OF FN14 RECEPTOR-TARGETED IMMUNOTOXINS FOR BREAST CANCER THERAPY

BC086135

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Background and Objectives: The TNF-related cytokine TWEAK is a pro-angiogenic and pro-inflammatory factor that acts via binding to a small cell surface receptor named Fn14. Previous studies have indicated that Fn14 is over-expressed in multiple human solid tumor types, including brain, breast, pancreatic, esophageal, colon and lung cancers. High Fn14 expression levels in breast tumors correlates with several poor clinical prognostic indicators and with the aggressive HER2+/ER- intrinsic subtype. Our recent *in vitro* studies have revealed that HER2 signaling in breast cancer cell lines can directly induce Fn14 gene expression and that Fn14 function may contribute to HER2-triggered cellular responses. In consideration of these findings we initiated studies to investigate whether we can develop Fn14-targeted immunotoxins with sufficient activity and specificity for potential use as breast cancer therapeutic agents.

Brief Description of Methodologies: We have developed two different types of Fn14-targeted immunotoxins. The first type, designated ITEM4-rGel, was produced by chemically conjugating the high-affinity anti-Fn14 monoclonal antibody ITEM4 to recombinant gelonin (rGel), a highly cytotoxic, ribosome-inactivating n-glycosidase. The second type of immunotoxin, designated $_{h}$scFvIT4/rGel/29, is more suitable for potential clinical use. It was generated as follows. First, a recombinant humanized single-chain version of ITEM4 was successfully produced. Second, we engineered a bivalent immunotoxin by constructing a fusion protein consisting of $_{h}$scFvIT4, rGel, and a COOH-terminal dimerization domain. Both types of immunotoxins were purified to homogeneity and then tested for Fn14 binding and cytotoxic activity *in vitro* using Fn14-positive or Fn14-negative cells.

Results to Date: Both the ITEM4-rGel conjugate and the $_{h}$scFvIT4/rGel/29 single-chain fusion protein bind to various Fn14-expressing tumor cells, including the MDA-MB-231 breast cancer cell line. Cytotoxicity studies showed that both agents were highly cytotoxic to Fn14-expressing cells (IC_{50} ranged from 0.8 pM-25 nM) and were 50 to 0.5 $x10^6$ fold more potent than free rGel. Minimum contact time studies showed that as little as 8 hr exposure achieved maximal cytotoxic effect. Additional studies are in progress to investigate the mechanistic basis for the observed cytotoxic activity and to evaluate whether these Fn14-targeted immunotoxins can inhibit tumor growth *in vivo.*

Conclusions and Potential Impact on Breast Cancer: These results indicate that an anti-Fn14 antibody can be used to deliver a cytotoxic molecule into Fn14-positive tumor cells. We propose that the $_{h}$scFvIT4/rGel/29 construct in particular may warrant further development as a novel therapeutic agent against a broad range of Fn14-positive solid tumor types, including HER2+ breast tumors with either intrinsic or acquired resistance to HER2-targeted drugs.

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